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(54) **Title:** NOVEL USE OF PROTON PUMP INHIBITORS

(57) **Abstract:** The present invention relates to a novel use of proton pump inhibitors, such as omeprazole, esomeprazole, lansoprazole, rabeprazole and pantoprazole. More closely, the invention relates to use of proton pump inhibitors (PPIs) for production of a drug for treatment or prevention of a MDR (multidrug resistance) condition, such as a condition associated with cancer treatment.

NOVEL USE OF PROTON PUMP INHIBITORS

Field of the invention

The present invention relates to a novel use of proton pump inhibitors, such as omeprazole, esomeprazole, lansoprazole, rabeprazole and pantoprazole. More closely, the invention relates to use of proton pump inhibitors (PPIs) for production of a drug for treatment or prevention of a MDR (multidrug resistance) condition. In other words, the invention relates to use of proton pump inhibitors in combination with a drug for treatment of and in eucaryotic cells in the purpose of decreasing development of resistance against said drug.

Background of the invention

Resistance to drugs is a major obstacle in the treatment of various diseases, such as resistance to cytotoxic agents in the treatment of cancer. Cellular resistance mechanisms have been extensively studied in cell lines, which have acquired drug resistance from exposure to increasing concentrations of a cytotoxic drug. A phenomenon often observed is that cells exposed to a single drug develop cross-resistance to several groups of cytotoxic drugs, with different chemical structures and mechanisms of action. This phenomenon has been termed multidrug resistance (MDR) and indicates a general cellular defence mechanism against toxic agent [1]. The classical MDR phenotype includes resistance to anthracyclines, vinca alkaloids, epipodophyllotoxins, taxanes and mitomycin C[2]. Early studies demonstrated that MDR was associated with decreased drug accumulation and increased expression of the membrane bound 170 kD P-glycoprotein (Pgp), coded for by the MDR1 gene. Pgp is believed to function as an ATP-dependent efflux protein which actively extrudes drugs from the tumor cell [1]. Pgp belongs to the ATP-Binding-Cassette (ABC) protein family, which also contains other proteins related to multidrug resistance, such as the Multidrug Resistance Protein (MRP) [3] and the recently discovered Breast Cancer Related Protein (BCRP) also known as Mitoxantrone resistance-associated transporter (MXR) [4].

In early 80's, Tsuruo and colleagues demonstrated that the calcium channel blocker verapamil greatly potentiated the activity of vincristine in vitro and in vivo and increased cellular

accumulation of the cytotoxic drug in an MDR leukemia cell line [5]. Since then, many investigators have focused on studies of pharmacological reversal of MDR with non-cytotoxic agents. A large number of such resistance modifiers have been identified, including calcium channel blockers, cyclosporins, phenothiazines, neuroleptics and cephalosporins. The resistance modifiers generally affect the drug accumulation in MDR cells, and many of them have been shown to competetively inhibit Pgp transport function in drug resistant cell lines [6, 7]. In clinical pilot phase trial both cyclosporins and calcium channel blockers have shown promising activity in myeloma and lymphoma whereas the results have been less impressive in solid tumors [8], [9].

The malaria parasite´s development of resistance to the drug chloroquine is a major threat to world health. Malaria is among the most significant sources of global disease burden, causing approximately two million deaths every year, primarily in young children and pregnant women. The drug chloroquine, since its availability in the late 1940s, has been the mainstay of efforts to treat and control the disease.
Resistance to chloroquine has, however, steadily spread since the 1960s from two foci, one in South America and one in South East Asia. Now resistance has spread through Africa, the global heartland of malaria mortality and there are very few effective drugs to take its place. A better understanding of the nature of this resistance could help us design better drugs in the future. There are several mechanisms suggested and there are also causes for reduced chloroquine uptake. A number of compounds, such as the so-called chloroquine-resistance-reversal agent verapamil, is capable of specifically enhancing the activity of chloroquine against chloroquine-resistant strains. Its mechanism of action is not clear though it appears to work by enhancing chloroquine uptake of the parasite. The mechanism of chloroquine uptake has recently been suggested to be related to a Na+/H+ exchanger [10].

Multidrug resistance pumps (MDRs) were first discovered in eucaryotic cells, and they confer drug resistance on cells ranging from human tumor cells, to a variety of pathogens. The mammalian P-glycoprotein and the ABC multidrug resistance associated protein (MRP), which act as multidrug efflux pumps, belong to two distinct subfamilies of ABC transporters. Although both contain duplicated ATP-binding cassette motifs, their transmembrane domains differ considerably. Homologs of both these components are found in every eucaryotic microbe tested, and several of them seem to play a role in multidrug resistance. ABC

transporters are also found in every parasite tested, so far, including *Plasmodium falciparum*. Resistance to chloroquine shares several phenotypic features with multidrug resistance in mammalian tumor cell lines and the malarial P-glycoprotein gene was designated 10 years ago (*pfmdr 1*). It has also been shown that certain mutations in *pfmdr 1* are associated with a loss in chloroquine sensitivity. The gene product Pgh 1 is located in the cell's food vacuole and by concentrating chloroquine into this vacuole, Pgh 1 may increase susceptibility to the drug.

Resistance to drugs is also a major obstacle in the treatment of bacterial infections. Both obligate and facultative intracellular infections present special therapeutic problems. They do not always respond readily to antibiotic therapy and often relapse after a course of antibiotic treatment.

Although a large number of resistance modifiers have been identified, in respect of several drugs against various diseases, there still remains a need of improved agents with MDR reversing ability.

Summary of the invention

The present inventors have found that proton pump inhibitors (PPIs) have MDR reversing ability. Thus, the inventors have discovered a completely new principle of MDR reversal. By using PPIs in combination with the desired drug the activity thereof can be greatly improved. The present invention relates to the use of PPIs in combination with a said drug in the purpose increasing the effect of the said drug in vivo. According to the present invention relates PPI is/are used in combination with a drug for treatment of and in eucaryotic cells in the purpose increasing the effect of a said drug. The use of PPI(s) is not restricted to any particular drug but is expected to be generally applicable for increasing the effect of treatment or prevention of any drug resistance.

For example, PPIs can potentiate the effect of vinca alcaloids, such as vincristine (vcr) in several human tumor cell lines and increase the activity of chloroquine inside the parasite in case of malaria.

The MDR reversing ability achieved with PPIs according to the invention, may be due to the fact that PPIs interfere with the plasma membrane efflux system that extrudes drugs from the cells to be treated. Alternative mechanisms may involve interference with subcellular drug distribution of cytotoxic drugs due to PPI effects on lysosomal drug trapping.

Thus, the invention relates to use of, or a method of using, proton pump inhibitor(s) for the production of a drug for treatment or prevention of a MDR (multidrug resistance) condition. Preferably, the PPI is omeprazole, esomeprazole, lansoprazole, rabeprazole or pantoprazole, or a combination thereof. The drug may comprise PPI(s) alone or in combination with the cytotoxic drug.

For example, the MDR condition is associated with cancer treatment with cytostatics, such as vincaalcaloids, such as vincristine and vinorelbine, taxanes, such as taxol and taxotere, tubuline inhibitors and tubuline stimulators. The use of the invention is especially advantageous when the MDR condition is associated with treatment of tumors with lymphatic origin.

According to another embodiment of the invention, the use of PPI is when the MDR condition is associated with treatment of a parasitic infection such as an infection by *Plasmodium falciparum* causing malaria. In this case the MDR condition is caused by an antiparasitic agent, such as chloroquine.

According to a further embodiment, the present invention also provides use of proton pump inhibitor(s) for the production of a drug for treatment or prevention of a resistance condition associated with treatment of a bacterial infection with antibacterial agent(s), such as antibiotic(s).

The resistance condition may be a MDR (multidrug resistance) condition.

According to the invention, the drug may comprise PPI(s) alone or in combination with antibiotic(s). Preferably, the PPI is omeprazole, esomeprazole, lansoprazole, rabeprazole or pantoprazole, or a combination thereof. A preferred example of an antibiotic is a macrolide, such as azitromycin.

The bacterial infection may be an intracellular or facultative intracellular infection and may be caused by, for example, *Legionella spp, Helicobacter pylori, Streptococcus pneumoniae, Staphylococcus spp, Streptococcus pyogenes, Chlamydia spp, Listeria monocytogenes.*

All the mentioned uses of PPI(s) according to the invention have the effect of increasing the effect of an in vivo administered drug. In other words, the use of PPI(s) of the invention gives a synergistic effect of a drug in vivo. This synergistic effect is for example seen in cancer patients treated with cytostatics.

According to a second aspect, the invention relates to a method of treating or preventing a MDR (multidrug resistance) condition, comprising administration of a pharmaceutical composition comprising proton pump inhibitor(s), PPI(s), to an individual. The administration of PPI(s) is also useful for increasing the effect of an in vivo administered drug. The pharmaceutical composition may comprise the drug and be administered simultaneously with the PPI(s). Alternatively, the pharmaceutical composition does not comprise the drug and in this case the drug is administered in association with the PPI(s) or at an interval before of after the PPI administration.

According to a third aspect, the invention provides a pharmaceutical composition comprising proton pump inhibitor(s) and a drug selected from cytostatics/anti-neoplastic agent(s); anti-parastic agents; and anti-bacterial agents. These agents may be selected from those mentioned above or other suitable agents.

Detailed description of the invention

The invention will be described more closely below in association with two non-limiting examples demonstrating the resistance modifying effect of PPIs in connection with different drugs.

Figure legends:

Fig. 1-3 show the concentration-response curves of the three cell-line pairs.
1a-g are U-937 GTB and U-937-vcr; **2a-g** are 8226/S and dox40;

3a-g are H69 and H69-AR. The graphs are presented as mean of 5-7 experiments ±SE. Incubation with only vcr or dox are presented as mean of 13-20 experiments ±SE.

Fig. 4a-b show the survival index % and the concentration-response curves of the cell-lines U937-GTB and U937-ver when incubated with taxotere or vinorelbine and lanzoprazole (2 and 10μg/ml).

Fig. 5a-c show the cytotoxicity of the PPIs and PSC alone on the cell-lines.

Fig 6 shows inhibitory effect of Lanzo in combination with chloroquine against *Plasmodium falsciparum.*

EXAMPLE 1: Effect of PPIs in cancer treatment

MATERIALS AND METHODS

Cell lines and maintenance

The lymphoma cell line U-937 GTB and its subline U-937-vcr, the small cell lung cancer cell line NCI-H69 (H69) and its subline H69-AR were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The myeloma cell line RPMI 8226/S (8226/S) and its doxorubicine (dox) resistant subline 8226/dox 40 (dox40) kind gifts from Dr W. S. Dalton, (Tuzon, AZ). U-937-vcr was selected for vincristine resistance, proposed to be tubulin associated [11]. H69-AR selected for dox resistance expresses the multidrug resistance protein, MRP [12], [3] while dox40, also selected for doxo resistance, expresses the P-glycoprotein 170 (Pgp).

Cells were cultured under standard cell culturing conditions using RPMI-1640 medium (Sigma, St Louis, MO), supplemented with 10% heat inactivated Fetal Calf Serum (FCS)(Hy Clone, Cramlington, UK), 2mM glutamine, 50μg/ml streptomycin and 60μg/ml penicillin (Hy Clone). In addition U-937-vcr were continuously grown in medium containing 10ng/ml vcr, dox40 was treated once a month with 0.24μg/ml dox. H69-AR alternately fed with drug-free medium and medium containing 0.46μg/ml dox. Growth and morphology were monitored twice a week.

Drugs and reagents

Adriamycin® (doxorubicine), Oncovin® (vincristine,) Taxotere® (docetaxel) and Navelbine® (vinorelbine) all purchased from commercial sources were obtained from the local hospital pharmacy. PSC 833 (PSC), kind gift from Novartis (Basel, Switzerland), were dissolved in ethanol at 1mg/ml and stored in -70°C. Fluoresceindiacetate (FDA; Sigma). Genistein (Sigma) was dissolved in DMSO to 10mg/ml and stored in -70° until use. Lansoprazole and omeprazole, powder (AstraZeneca and Takeda Chemical Company, LTD) were stored in +4° C protected from light. New stock solutions, 1mg/ml dissolved in ethanol, were freshly prepared made for each plate batch. All other reagents were of analytical grade and obtained from commercial sources.

Cytotoxicity assay

The assay used for detection of drug activity is called Fluorometric Microculture Cytotoxicity Assay (FMCA) [13]. It is a 96-well microtiterplate total cell kill assay, based on detecting fluorescence in cells with intact cell membranes. After 72 hrs. incubation with the compounds of interest nonfluorescent FDA is added to the wells. FDA is hydrolysed intracellulary in living cells by nonspecific esterases into fluorescent fluorescein. The fluorescence is directly proportional to the amount of living cells in each well. It is described briefly below. Ninetysix-well microtitre plates (Nunc, Roskilde, Denmark), flatbottomed (for H69 and H69-AR) and V-shaped (for the four other cell lines), were prepared with drugs at ten fold dilution steps in each row. For dox and vcr the highest concentrations were 10 and 0.5 µg/ml respectively. Plates were stored in -70° C no longer than three months until use.

On day one 180 µl cell suspension (0.27×10^5 cells/ml for H69 and H69-AR and 1.1×10^5 cells/ml for the other four cell lines) were seeded into the premade plates, using a pipetting robot, Pro/Pette II (Perkin Elmer, Norwalk, CT). Six wells with phosphate buffered saline (PBS) and cell suspension served as control and ten wells recieved only medium without cells served as blank. The plates were incubated for 72 hours in 37° C, 5% CO2. At the end of the incubation period the plates were centrifuged for 5 minutes, 200g. The medium in the V-shaped plates were removed by aspiration using a microplate washer, Multiwash, (Dynatech Laboratories, Chantilly, VA) and 200µl PBS was added using the same washer. The flatbottomed plates were flicked by hand and 200µl PBS were added with the above mentioned plate washer. After a repeated wash step, 100µl HEPES buffer containing FDA to

a final concentration of 10μg/ml were added to each well. The plates were incubated for 45 minutes at 37° C and then the generated fluorescence in each well were detected with a microtiterplate spectrofluorometer, Fluoroscan II, (Labsystems OY., Helsinki, Finland) excitation and emission filters set to 480 and 530 nm, respectively.
The results are presented as survival index (SI), defined as fluorescence in test wells in percent of control wells with blank values subtracted. All calculations were made with custom made software, Microsoft Excel, (Microsoft, Redmond, WA) using a MacIntosh computer (Apple Computer, Cupertino, CA).

Quality control

A pre-test viability of >90% cell viability prior to experiments, as judged by trypan blue exclusion test and fluorescence in control wells more than five times the mean blank values was required. Furthermore a coefficient of variation of less than 30% in the triplicate wells was also required.

RESULTS

FMCA

Five plate configurations were used; vcr-lansoprazole 2 and 10μg/ml, vcr-omeprazole 2 and 10μg/ml, vcr-PSC 1 and 3μg/ml, dox-lansoprazole 2 and 10μg/ml and dox-omeprazole 2 and 10μg/ml.

Figures 1-3 shows the concentration-response curves of the three cell-line pairs. 1a-g are U-937 GTB and U-937-vcr. 2a-g are 8226/S and dox40, 3a-g are H69 and H69-AR. The graphs are presented as mean of 5-7 experiments ±SE. Incubation with only vcr or dox are presented as mean of 13-20 experiments ±SE.

Table 1 shows IC50 values for the six cell lines. IC50 is defined as the drug concentration giving a SI of 50% compared to untreated control cells.
Figures 4b shows the concentration-response curves of cell-lines incubated with taxotere and vinorelbine, respectively, and lanzoprazole 2 and 10μg/ml

Figures 5a-c shows the cytotoxicity of the PPIs and PSC alone on the cell-lines.

U-937 GTB and U-937-vcr

Figure 1 a-g show concentration response curves for U-937 GTB and U-937-vcr.

At 10 µg/ml both lanzo and omeprazole decreased the IC50 values 4,5 and 6.5 fold, respectively in both cell lines. At 2 µg/ml lanzo a slight decrease in IC50 value was observed in U-937-vcr and for omeprazole there was a slight increase in IC50 values for vcr in both cell lines. (not significant)

When lanzo and omeprazole were incubated with dox no significant change in IC50 values were observed compared to dox alone. PSC was included as a reference compound for a potent PGP and a weak MRP inhibitor. At 1 and 3 µg/ml a 3-250 fold decrease of IC50 in the cell lines were observed.

8226/S and dox40

Figure 2 a-g shows concentration response curves for 8226/S and dox40.

IC50 was halved in 8226/S when vcr was co-incubated with lanzo or omeprazole at 10 µg/ml. At 2 µg/ml no significant change in IC50 was observed in 8226/S. Dox40 produced no IC50 value for vcr and vcr+omeprazole 2, IC74 and IC58 were 0.5 µg/ml. IC50 values for vcr+lanzo 2 and 10 and vcr+omeprazole 10 were 0.3 and 0.2 µg/ml. When lanzo and omeprazole were incubated with dox no significant change in IC50 values were observed compared to dox alone. PSC was included as a reference compound for a potent PGP and a weak MRP inhibitor. At 1 and 3 µg/ml a 3-250 fold decrease of IC50 in the cell lines were observed.

H69 and H69-AR

Figure 3 a-g shows concentration response curves for H69 and H69-AR.

Co-incubation with vcr and 10 µg/ml of lanzo or omeprazole a gave 2-5 fold decrease in IC50 was observed in H69-AR. At 2 µg/ml no clear effect was observed in H69-AR. For H69 no potentiating effect was noted at all. When co-incubated with dox a 2-fold decrease of IC50 was observed for lanzo 10µg/ml in H69-AR (not significant). PSC was included as a reference compound for a potent PGP and a weak MRP inhibitor. At 1 and 3 µg/ml a 2-250 fold decrease of IC50 in the cell lines were observed.

Table 1 IC_{50} values.

	U-937 GTB	U-937-vcr	8226/S	dox40	H69	H69-AR
vcr	0.009	0.13	0.008	0.5(IC74)	0.01	0.1
vcr+lanso 2	0.01	0.08	0.02	0.3	0.01	0.05
vcr+lanso 10	0.002	0.02	0.003	0.3	0.01	0.02
vcr+ome 2	0.02	0.1	0.02	0.5(IC58)	0.01	0.05
vcr+ome 10	0.002	0.02	0.005	0.2	0.005	0.02
vcr+PSC 1	0.001	0.001	0.002	0.003	0.003	0.02
vcr+PSC 3	0.00004	0.00005	0.0002	0.0002	0.0006	0.01
dox	0.03	0.2	0.1	4.4	0.05	0.6
dox+lanso 2	0.05	0.3	0.2	7.7	0.04	0.4
dox+lanso 10	0.05	0.3	0.2	4.4	0.03	0.3
dox+ome 2	0.03	0.2	0.1	3.4	0.04	0.4
dox+ome 10	0.03	0.2	0.1	3.4	0.04	0.6

Taxotere and PPI; and vinorelbine and PPI

Figure 4a shows the effect of lanzoprazole on vinorelbine and taxotere induced cytotoxicity. As appears in the figure, SI% (survival index) is markedly reduced by the addition of PPI.

Figure 4b shows the effect of lanzoprazole on vinorelbine and taxotere induced cytotoxicity in U-937 GTB and U-937-vcr cells. As appears in the figure, PPI's have a significant effect on taxotere and vinorelbine induced cytotoxicity in U-937-vcr-cells.

Cytotoxic activity of the PPIs and PSC

Figures 5a-c shows the cytotoxicity of the PPIs and PSC alone on the cell-lines.

SI of U-937 GTB and U-937-vcr varied between 99-119% when incubated with the PPIs alone. PSC decreased SI dose-dependently in U-937 GTB and U-937-vcr.

SI of 8226/S and dox40 varied between 90-113% when incubated with the PPIs alone. At 3 (g/ml of PSC, SI was 76 % compared to untreated dox40.

SI in H69 and H69-AR varied between 73-110% when incubated with the PPIs alone. A SI of 76 % was observed in H69-AR when incubated with PSC.

Discussion

The lower concentration of both lanzo and omeprazole both increased and decreased IC50 although the differences were small. Co-incubation with lansoprazole and omeprazole at 10

μg/ml increased the sensitivity towards vcr in all cell lines but H69. In general, an increased sensitivity of 50 % to a cytotoxic drug or more when co-incubated with a modulating compound is regarded of interest from a clinical point of view. PSC is regarded as a potent Pgp inhibitor and clinical trials have been performed in various tumour types, although it is not registered as PGP inhibitor. The concentrations used in this study are on the upper limits of what has been achieved in clinical trials on patients. PSC appeared also more toxic towards U-937 GTB in comparison to the parental cell line U-937 GTB. This is in accordance with earlier findings that U-937-vcr is more sensitive to the related compound CsA than the parental U-937 GTB [10].

Regarding the cytotoxicity of the modulators alone. A SI of 10-15 % lower than untreated control is not to be regarded as toxic. The SI observed in H69-AR when incubated with lanzo 10 μg/ml was lower, 73 % but still probably not of clinical interest.

A SI above 100 % such as for U-937 GTB for example is probably due to assay variability but may also indicate an unspecific growth stimulation or a stimulation of the intracellular esterases responsible for the hydrolysis of FDA to fluorescein.

Since the Pgp and MRP expressing sub-lines dox40 and H69-AR were affected by PPI modulation, this may indicate that lansoprazole and omeprazole affects the activity of the efflux proteins Pgp and MRP. The more pronounced PPI interaction with tubulin-active agents in lymphocytic cells may be due to specific interference with tubuline associated MDR. The resistance mechanism may involve alternations in subcellular drug distribution caused by PPI effects on lysosomal drug trapping.

In conclusion, coincubation with vincristine and lansoprazole or omeprazole increased the vincristine sensitivity up to six times in U-937 GTB, U-937-vcr, 8226/S, dox40 and H69-AR. Use of PPI's according to the invention can also increase the sensitivity of other vincaalcaloides, taxanes and tubulin active agents.

EXAMPLE 2: Effect of PPIs in the treatment of parasitic infections

MATERIALS AND METHODS

***P. falciparum* strain and culture conditions**

The chloroquine resistant *P. falciparum* strain FCR 3 S1 was kept in continuos culture in RPMI-hepes medium supplemented with 10% AB serum. A ^{3}H-hypoxanthine incorporation assay for screening of inhibitory effect of different drug compounds was used [14, 15]. The parasite was diluted in the medium to a parasitemia of 0.2% and a hematocrite of 2%.

Inhibition assay

Lanzoprazole (Takeda chemical industries LTD, Osaka, Japan) were dissolved in DMSO and diluted in RPMI to final concentrations of 2, 5, 10 and 20 μg/ml. Chloroquine was used in concentrations from 10 μg/ml down to 2 ng/ml (fig 7). The effect of lanzoprazole was tested with and without the addition of chloroquine. DMSO alone was used as control. The microtiter plates were incubated in a candle jar at 37 °C for 24 hours to allow inhibition of the proliferation. ^{3}H-hypoxanthine at a concentration of 0.5 μCi diluted in RPMI medium was added to each well and the culture was continued for another 18 hours. Finally, the parasite cultures were harvested on filter papers using an automatic cell harvester. The incorporated radioactivity was measured in a microbeta counter after addition of scintillation fluid. Results are shown as mean inhibition of five polymerisation assays.

RESULTS

Dose dependent reversal of resistance

Lanzoprazole alone had an mean inhibitory effect at the highest concentration (20 μg/ml) comparable to inhibition of the *P. falciparum* proloferation in combination with chloroquine at 2 ng/ml. Lanzoprazole alone or in combination with chloroquine had an inhibitory effect on the resistant strain FCR3 S1 and the synergistic effect was clearly dose dependent (Fig 6). The cut-off level for the synergistic effect was shown when the chloroquine concentration reached 32 ng/ml. A dose dependent effect was also shown for concentration of lanzoprazole (Fig 6). DMSO alone had no inhibitory effect.

DISCUSSION

It may be difficult to prove the direct involvement of lanzoprazole as a potential modifier of chloroquine transport or as maintaining cellular pH subsequent to chloroquine uptake. The wild-type *pfmdr1* gene product Pgh1 is located in the vacuole, which is the site of action of chloroquine. Pgh1 contributes to the acidification of the vacuole by pumping either directly or indirectly a pronated compound within the valuole. Being a weak base, chloroquine may accumulate into vacuoles in which the pH is decreased. Mutations in *pfmdr1* would prevent such a decrease in pH, hence less chloroquine would accumulate at the site of action. Lanzoprazole may maintain low pH-levels by a so far unknown mechanism to concentrate chloroquine into the vacuoles and by this revert resistance. Another possible mechanism could be that lanzoprazole interferes with a plasma membrane efflux system. By acting at this level, a synergistic effect between chloroquine and lanzoprazole will appear and thus, the proton pump inhibitor may act as a resistance modulator. Researcher are seeking additional, non-pfmdr1 chloroquine resistance genes, and one such candidate gene segregates with active efflux of the drug.

To sum up, our results show that lanzoprazole has a synergistic effect in combination with chloroquine in the inhibition assay described above. Interestingly, lanzoprazole alone also had an inhibitory effect. Both effects were dose dependent.

EXAMPLE 3: Effect of PPI's in the treatment of bacterial infections

The invention also describes intracellular killing of bacteria by azitromycin - a macrolide with a high degree of intracellular accumulation - in cells expressing resistance to cytotoxic drugs. Human derived cell-lines were used, with varying degree of multidrug resistance. Drugs that might interfere with antibiotic efflux and thus could act as resistance modulators were studied. Since proton-pump inhibitors (PPIs) such as omeprazole, lansoprazole and similar substances seem to have a synergistic effect in treatment of *H. pylori* infections with antibiotics, these drugs were tested in combination with azithromycin in order to study the efficacy of intracellular killing of *H. pylori* in cells with varying degrees of drug resistance.

Material and methods

Preparation of bacteria

H. pylori, clinical strain 88-23 (kindly provided by M. Blaser, Nashville, TN, USA), was grown on Colombia agar plates (Colombia II agar Base BBL®, Becton-Dickinson and Company, Cockeysville, MD, USA supplemented with 10% horse serum and 8.5% horse blood). Growth was performed at 37°C in a humid atmosphere under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2). Prior to infection, the bacteria were cultured for 36 h in Brucella Broth (10 g/l Bacto® Peptone, (Difco Laboratories, Detroit, MI, USA), 5 g/l Lab lemco Powder (Oxoid, Unipath LTD, Basingstoke, Hampshire, England), 10 g/l Dextrose, 5 g/l NaCl, 1 % Isovitalex BBL® (Becton-Dickinson and Company, Cockeysville, MD, USA)) with 10% fetal calf serum. The pH of the broth was adjusted to 6.0. The purity of the *H. pylori* cultures was verified by Gram staining and by biochemical characteristics i.e. positive in urease, catalase, and oxidase tests.

Cell culture

The established human monoblastold cell line U-937 GTB [12] and two vincristine (Vcr)-resistant sublines [13] - U-937Vcr_{10} and U-937Vcr_{100} - were cultured in a medium consisting of RPMI 1640 (Gibco BRL, Life Technologles™, Paisley, Scotland), 10% fetal calf serum, 4 mM glutamine, 20 mM HEPES (N-2-hydroxyethylepiperazine-N-2-ethanesulfonic acid) and 0.98% $NaHCO_2$. The cultures were incubated in 37°, with medium change and subcultivation twice a week. Prior to infection by *H. pylori,* the cells were diluted to a density of 1-2x10^5 cells/ml and seeded in 24-well plates, 1 ml of cell suspension per well. After seeding, the cells were allowed to grow for 36h in 37° in a humidified 5% CO_2-in-air atmosphere.

Exposure of *cells to H. pylori*

H. pylori broths were centrifuged for 15 min at 1000 x g. The bacterial pellet was resuspended in RPMI-medium to a final concentration of about 1x10^8 bacteria/ml and 10 µl of the suspension was added to each well containing cells. Internalization was allowed for 12 h in 37° in an atmosphere with 5% CO_2. Extracellular *H. pylori* was eradicated by adding gentamicin (Gensumicin®, Roussel, Denham, Uxbridge, UK) to a final concentration of 50 mg/l in the wells and incubating the exposed cells for 2 h.

Drug treatment

Azithromycin (Pfizer Inc., New York, USA) was added to the wells to a final concentration of 10 mg/l with or without either omeprazole (Astra, Stockholm, Sweden) at a concentration of 20 mg/l, or lansoprazole (Takeda chemical industries LTD, Osaka, Japan) at 2 mg/l. One control group was incubated with medium only. At 0, 4 and 24 h, cells from each group were transferred to Eppendorf tubes. The cells were washed twice with PBS, followed by 10 minutes lysis with distilled water. After centrifugation at 15800 x g for 5 minutes, the pellets, consisting of bacteria and cell debris, were dissolved in 1 ml of PBS. One hundred microliters from each sample was plated on Colombia agar plates and incubated for 3 days before the colonies, i.e. the viable intracellular bacteria, were counted.

Results

Dose dependent intracellular killing of H. pylori by azitromycin.

Cell lines selected in different concentration of Vincristine, and exhibiting different MDR phenotypes were used in an intracellular infection assay. After incubation with *H. pylori,* the cells were treated with gentamicin to eradicate extracellular bacteria. Azitromycin (10mg/l) was added to the medium, and surviving bacteria were counted at regular intervals. The intracellular survival of *H. pylori* was approximately a 100-fold higher in the resistant cells, with the bacteria totally protected at the azitromycin concentration used (10mg/l). PPI alone had no effect on the intracellular bacteria (data not shown). Adding lansoprazole or omeprazole made the bacteria susceptible to azitromycin to the same degree as those infecting the non-MDR U-937 GTB cell line. Furthermore, at 4 hours a potentiating effect of PPI on azitromycin was seen in the sensitive U-937 GTB cells.

Extracellular killing of H. pylori using a combination of azitromycin and PPI.

To examine weather there was a direct effect of PPIs on bacterial killing, or a synergistic or additive effect of PPIs and azitromycin, an experiment was performed were extracellular bacteria were treated. No positive or negative effect of adding PPI to azitromycin could be shown.

Discussion

Antibiotic resistance in intracellular organisms can be due not only to factors in the infecting microorganisms, but also to properties of the host cells. Several known mechanisms of *in*

vitro resistance to cytotoxic drugs could also be applicable for antibiotics. We have shown that *H. pylori* can be protected from the actions of certain antibiotics by infected cells displaying different patterns of drug resistance.

The efficacy of intracellular killing of *H. pylori* was decreased both in cells overexpressing Pgp and in non-Pgp expressing multidrug resistant cells. The U-937Vcr$_{10}$ cells were also not expressing the Multidrug Resistance Protein (MRP) (data not shown), and showed a resistance profile differing from other types of MDR cells with known mechanisms of resistance. It has previously been shown that erythromycin has reduced intracellular activity in Pgp-expressing cells. Our findings extend this observation to a non-Pgp-expressing MDR cell-line.

We also show that proton-pump inhibitors could reverse the resistance to intracellular killing by azitromycin in these cells. This effect was only seen in intracellular infection with *H. pylori*, and not on axenically grown bacteria, implying an effect of PPI on host cell mechanisms of resistance. The mechanism of resistance reversal of PPIs on cells with non-Pgp mediated resistance might be by interfering with the transport of azitromycin in or out of the cell, or by changing the intracellular pH, which in turn might alter the efficacy of azitromycin.

The fact that MDR cells may render intracellular bacteria inaccessible to certain antibiotics might have important implications for the persistence of these infections. If cells expressing MDR are infected *in vivo*, this could result in chronic, therapy-resistant infections. Whether this is the case in *H. pylori* infections is under debate, but we have shown that this is theoretically possible. Observations both in vitro and in vivo have shown that *H. pylori* is capable of invading epithelial cells. The sanctuary that provides a reservoire for *H. pylori* to reproduce the stomach after unsuccessful therapy is still unknown but one possibility is that *H. pylori* is also an intracellular organism with clinical consequences. Thus, by having a protective strategy against extracellular antibacterial activity successful eradication requires addition of antibiotics with intracellular activity such as macrolides. Our observation could explain the seemingly synergistic effect of macrolides and proton-pump inhibitors in the clinical situation when treating *H. pylori*- infected patients.

References

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CLAIMS

1. Use of proton pump inhibitor(s) for the production of a drug for treatment or prevention of a MDR (multidrug resistance) condition.

2. Use according to claim 1, wherein the proton pump inhibitor is omeprazole, esomeprazole, lansoprazole, rabeprazole or pantoprazole, or a combination thereof.

3. Use according to claims 1 or 2, wherein the drug, besides PPI(s), also comprises the resistance causing drug.

4. Use according to claims 1, 2 or 3, wherein the MDR condition is associated with cancer treatment with cytostatics/anti-neoplastic agents.

5. Use according to claim 4, wherein drug comprises PPI(s) and cytostatics/anti-neoplastic agent(s).

6. Use according to claim 5, wherein the drug is selected from vincaalcaloids, taxanes and tubuline active agents.

7. Use according to any of the claims 4-6, wherein the MDR condition is associated with treatment of lymphatic tumours.

8. Use according to claims 1, 2 or 3, wherein the MDR condition is associated with treatment of a parasitic infection with an antiparasitic agent.

9. Use according to claim 8, wherein the drug comprises PPI(s) and antiparasitic agent(s).

10. Use according to claims 8 or 9, wherein the parasitic infection is by *Plasmodium falciparum* causing malaria.

11. Use according to claims 9 or 10, wherein the antiparasitic agent is chloroquine.

12. Use according to claims 1, 2 or 3, wherein the MDR condition is associated with treatment of a bacterial infection with an antibacterial agent.

13. Use according to claim 12, wherein the drug comprises PPI(s) and antibacterial agent(s).

14. Use according to claims 12 or 13, wherein the bacterial infection is by *Legionella spp, Helicobacter pylori, Streptococcus pneumoniae, Staphylococcus spp, Streptococcus pyogenes, Chlamydia spp, Listeria monocytogenes.*

15. Use according to claims 12, 13 or 14, wherein the antibacterial agent is antibiotic(s).

16. Use according to claim 15, wherein the antibiotic is a macrolide.

17. Method of treating or preventing a MDR (multidrug resistance) condition, comprising administration of a pharmaceutical composition comprising proton pump inhibitor(s) to an individual.

18. Pharmaceutical composition comprising proton pump inhibitor(s) and a drug selected from cytostatics/anti-neoplastic agent(s); anti-parastic agents; and anti-bacterial agents.

Fig. 1

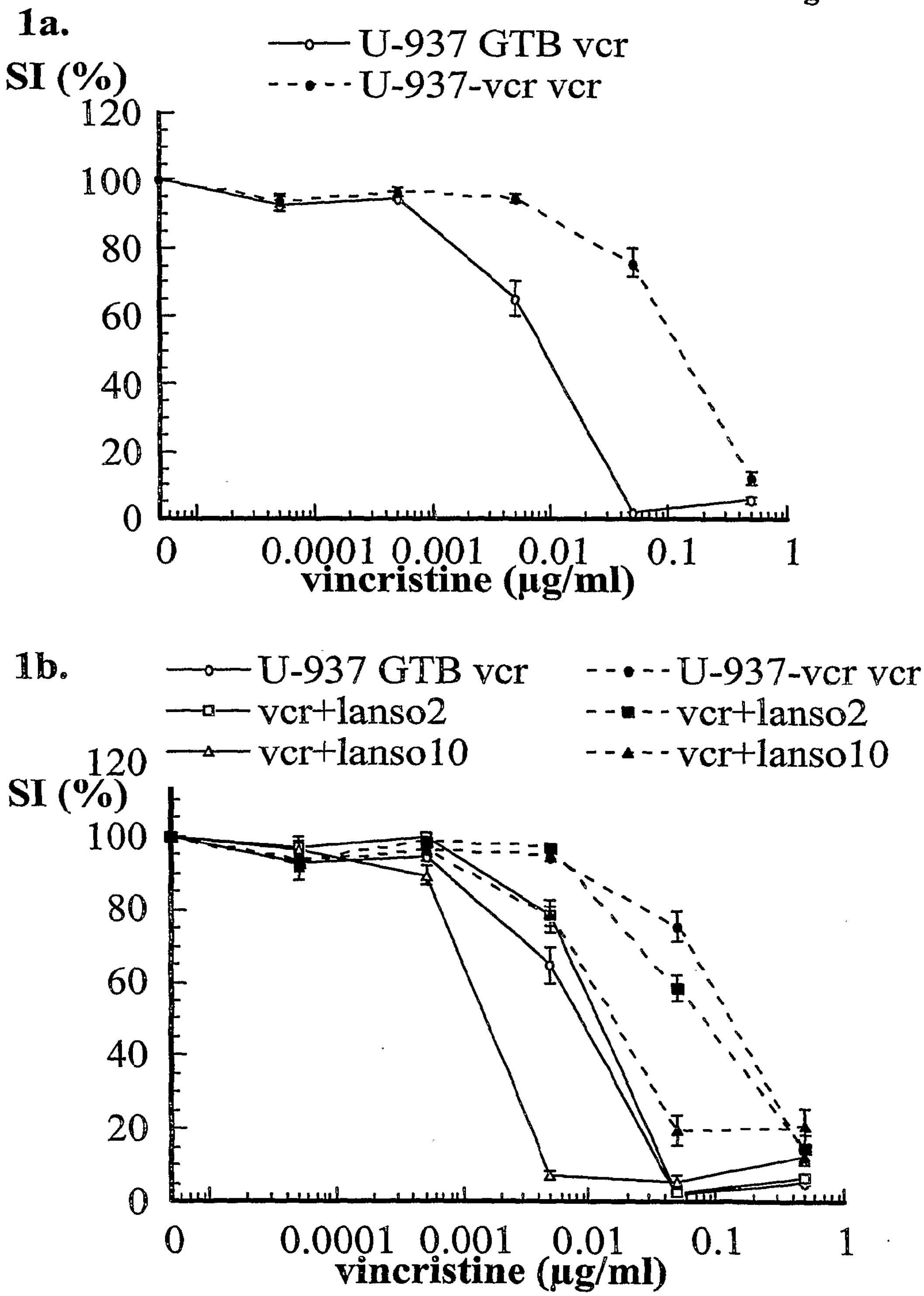

1a.
U-937 GTB vcr
U-937-vcr vcr
SI (%)
120
100
80
60
40
20
0
0
0.0001
0.001
0.01
0.1
1
vincristine (µg/ml)
1b.
U-937 GTB vcr
U-937-vcr vcr
vcr+lanso2
vcr+lanso2
vcr+lanso10
vcr+lanso10
SI (%)
120
100
80
60
40
20
0
0
0.0001
0.001
0.01
0.1
1
vincristine (µg/ml)

Fig. 1 cont.

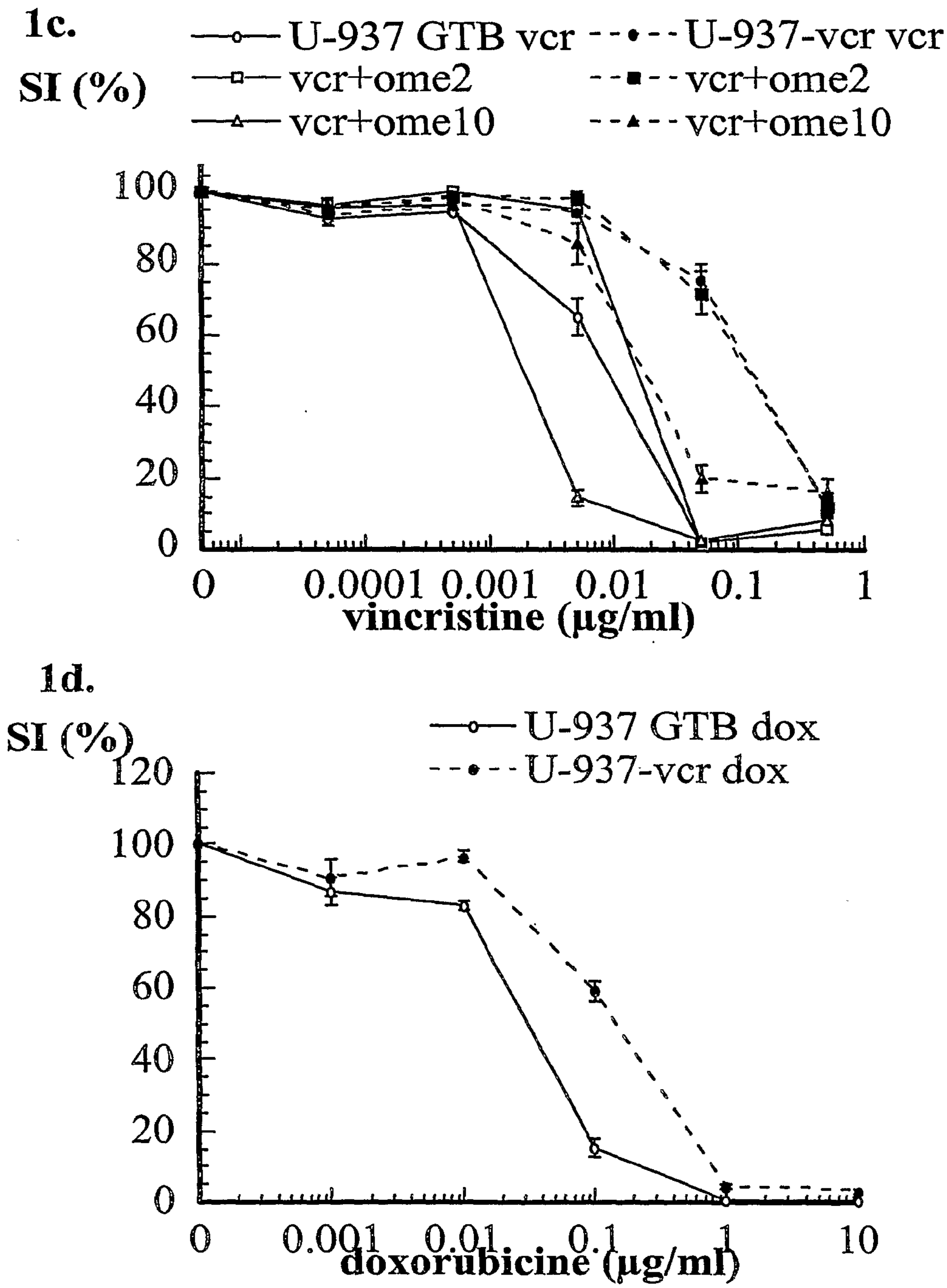

1c.
SI (%)
U-937 GTB vcr
U-937-vcr vcr
vcr+ome2
vcr+ome2
vcr+ome10
vcr+ome10
100
80
60
40
20
0
0
0.0001
0.001
0.01
0.1
1
vincristine (µg/ml)
1d.
SI (%)
U-937 GTB dox
U-937-vcr dox
120
100
80
60
40
20
0
0
0.001
0.01
0.1
1
10
doxorubicine (µg/ml)

Fig. 1 cont.

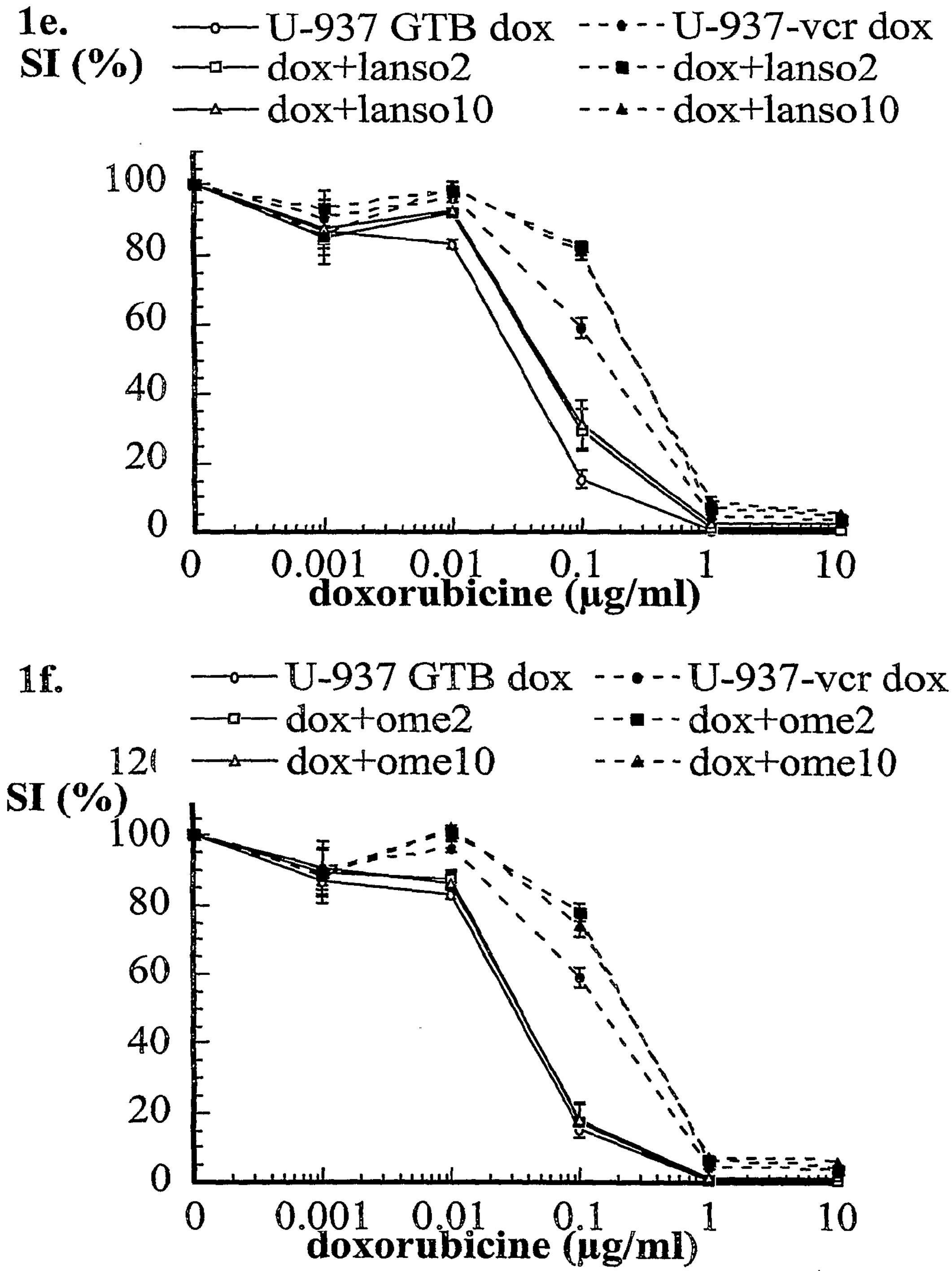

1e.
SI (%)
U-937 GTB dox
dox+lanso2
dox+lanso10
U-937-vcr dox
dox+lanso2
dox+lanso10
100
80
60
40
20
0
0
0.001
0.01
0.1
1
10
doxorubicine (µg/ml)
1f.
U-937 GTB dox
dox+ome2
dox+ome10
U-937-vcr dox
dox+ome2
dox+ome10
120
SI (%)
100
80
60
40
20
0
0
0.001
0.01
0.1
1
10
doxorubicine (µg/ml)

Fig. 1 cont.

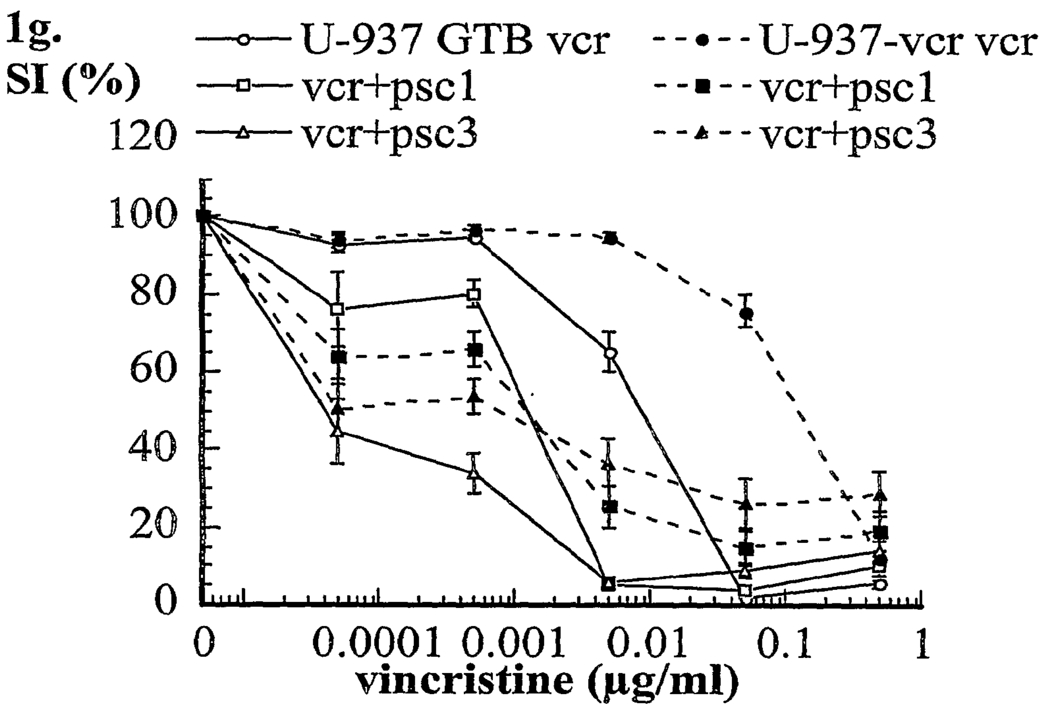

1g.
SI (%)
U-937 GTB vcr
vcr+psc1
vcr+psc3
U-937-vcr vcr
vcr+psc1
vcr+psc3
120
100
80
60
40
20
0
0
0.0001
0.001
0.01
0.1
1
vincristine (µg/ml)

Fig. 2

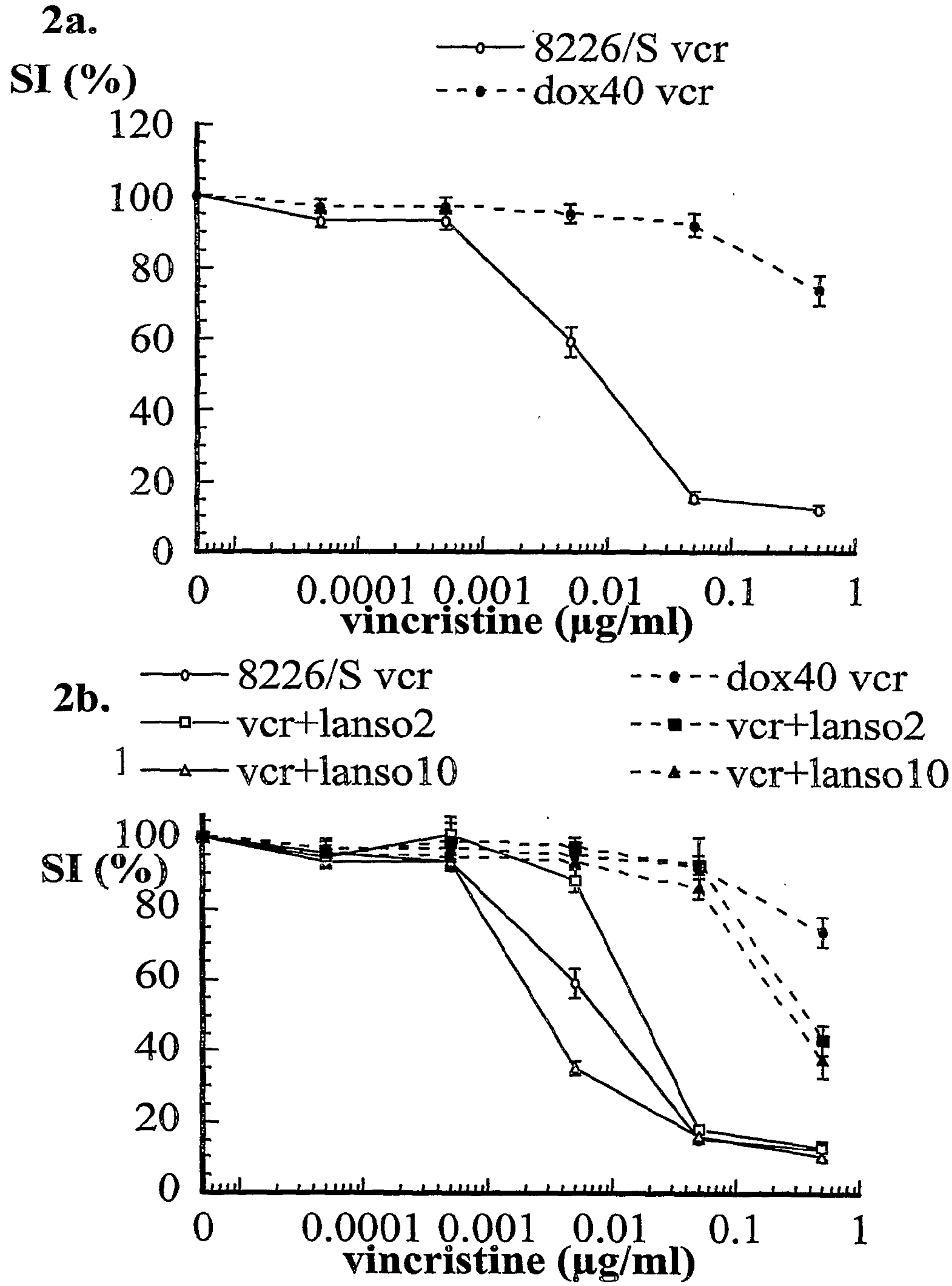

2a.
SI (%)
8226/S vcr
dox40 vcr
120
100
80
60
40
20
0
0
0.0001
0.001
0.01
0.1
1
vincristine (µg/ml)
2b.
8226/S vcr
vcr+lanso2
vcr+lanso10
dox40 vcr
vcr+lanso2
vcr+lanso10
SI (%)
100
80
60
40
20
0
0
0.0001
0.001
0.01
0.1
1
vincristine (µg/ml)

Fig. 2 cont.

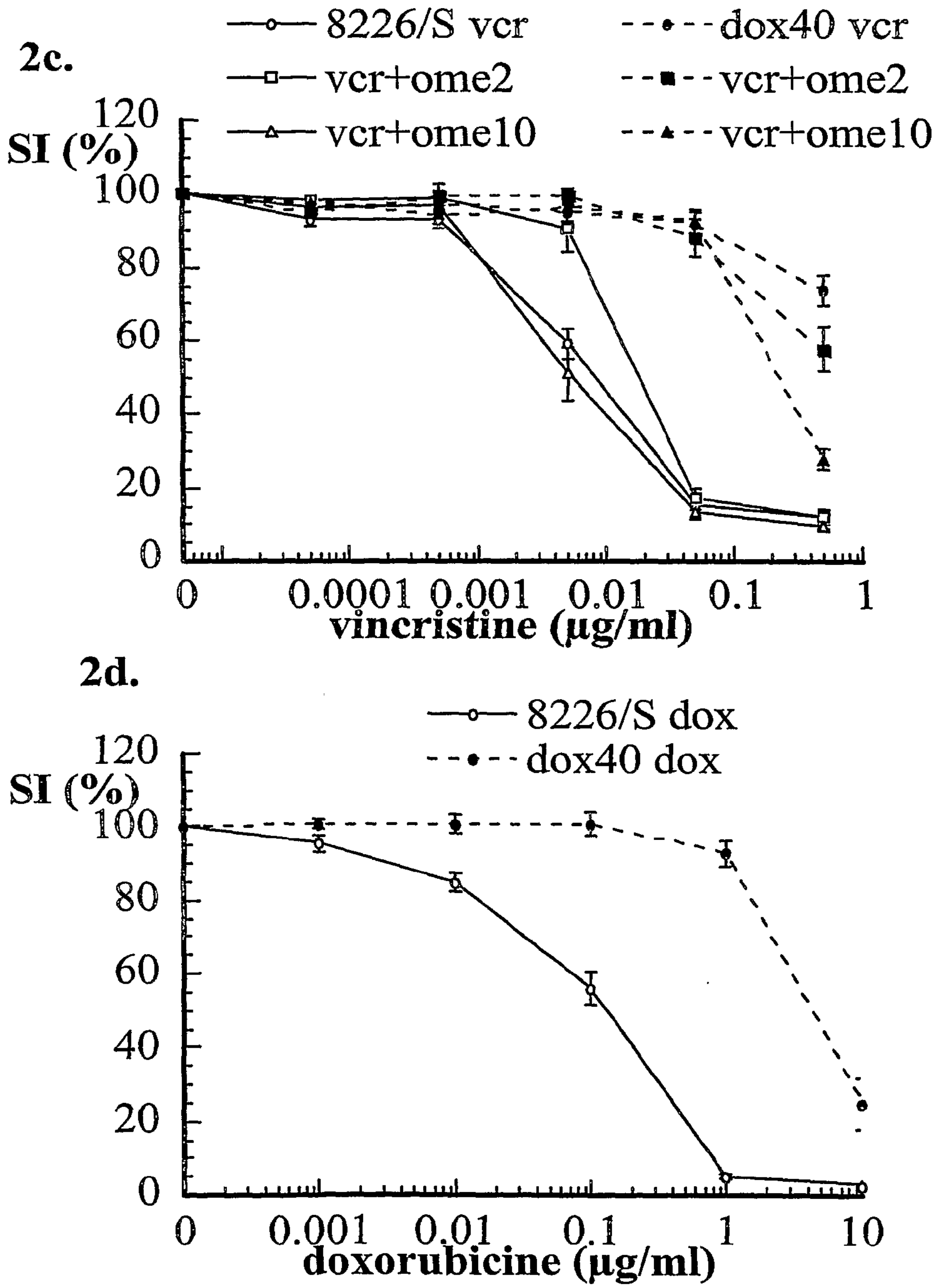
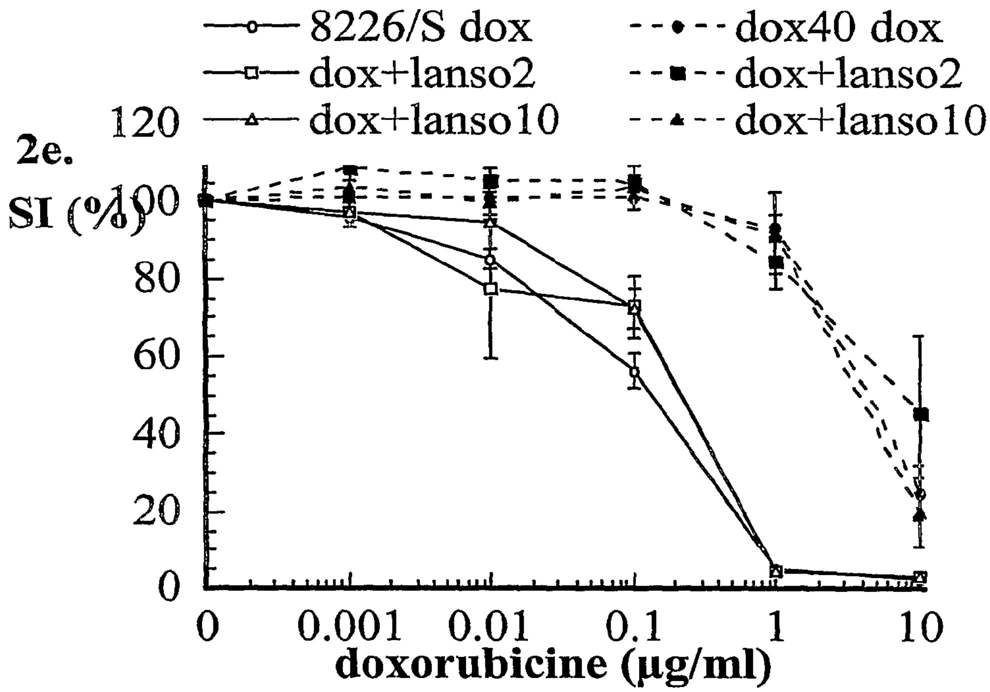

2c.
8226/S vcr
vcr+ome2
vcr+ome10
dox40 vcr
vcr+ome2
vcr+ome10
SI (%)
120
100
80
60
40
20
0
0
0.0001
0.001
0.01
0.1
1
vincristine (µg/ml)
2d.
8226/S dox
dox40 dox
SI (%)
120
100
80
60
40
20
0
0
0.001
0.01
0.1
1
10
doxorubicine (µg/ml)

Fig. 2 cont.

2e.



2f.

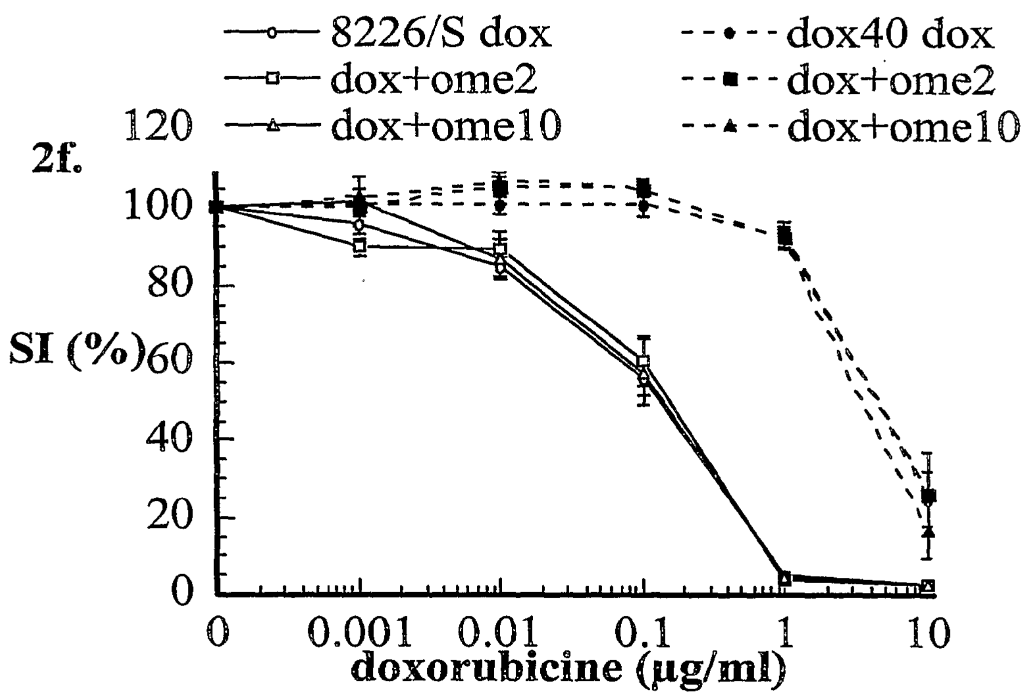


Fig. 2 cont.

2g.

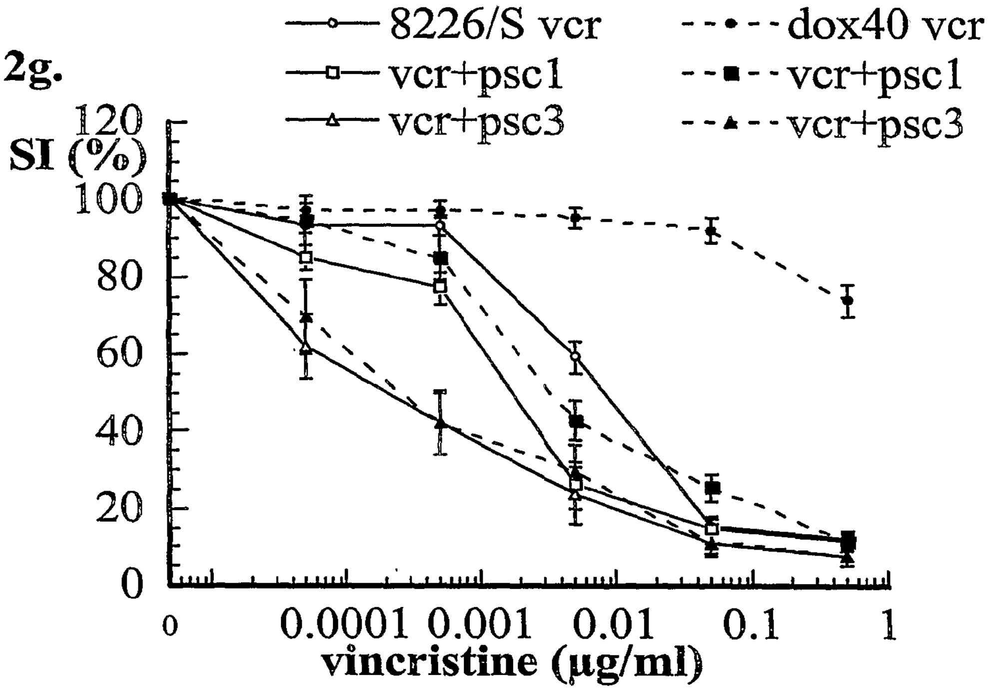
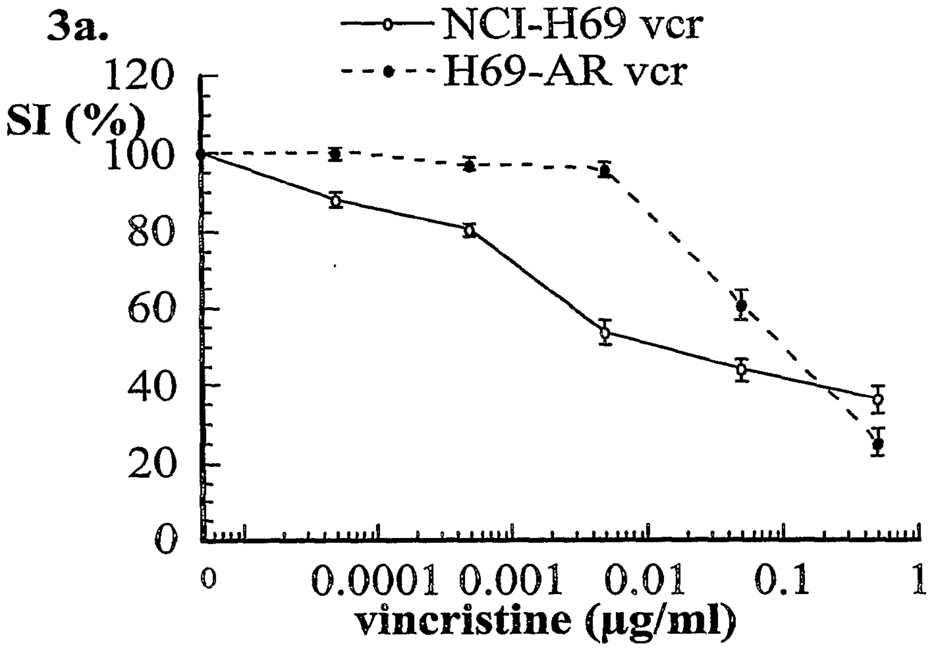

8226/S vcr
vcr+psc1
vcr+psc3
dox40 vcr
vcr+psc1
vcr+psc3
SI (%)
120
100
80
60
40
20
0
0
0.0001
0.001
0.01
0.1
1
vincristine (µg/ml)

Fig. 3

3a.


NCI-H69 vcr
H69-AR vcr
SI (%)
120
100
80
60
40
20
0
0
0.0001
0.001
0.01
0.1
1
vincristine (µg/ml)

3b.

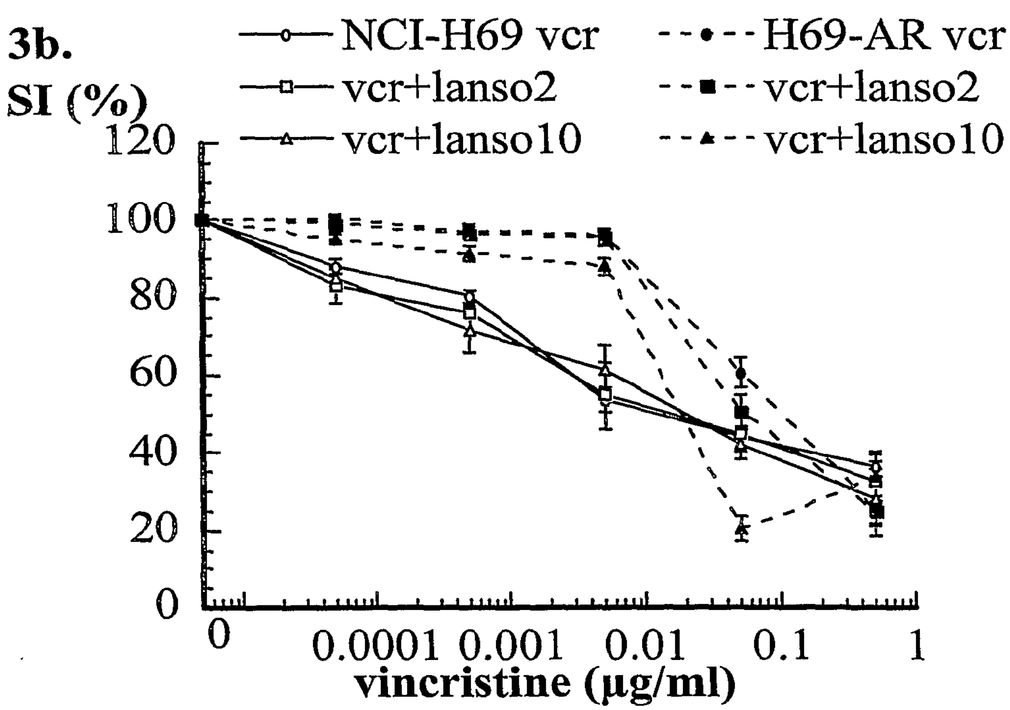
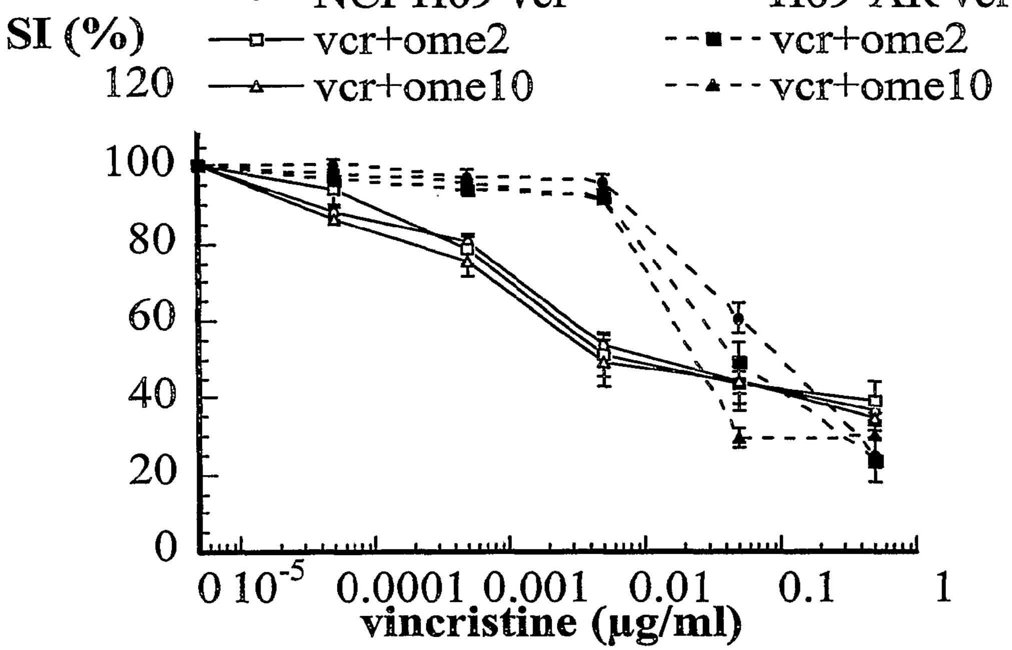

NCI-H69 vcr
vcr+lanso2
vcr+lanso10
H69-AR vcr
vcr+lanso2
vcr+lanso10
SI (%)
120
100
80
60
40
20
0
0
0.0001
0.001
0.01
0.1
1
vincristine (µg/ml)

Fig. 3 cont.

3c.



3d.

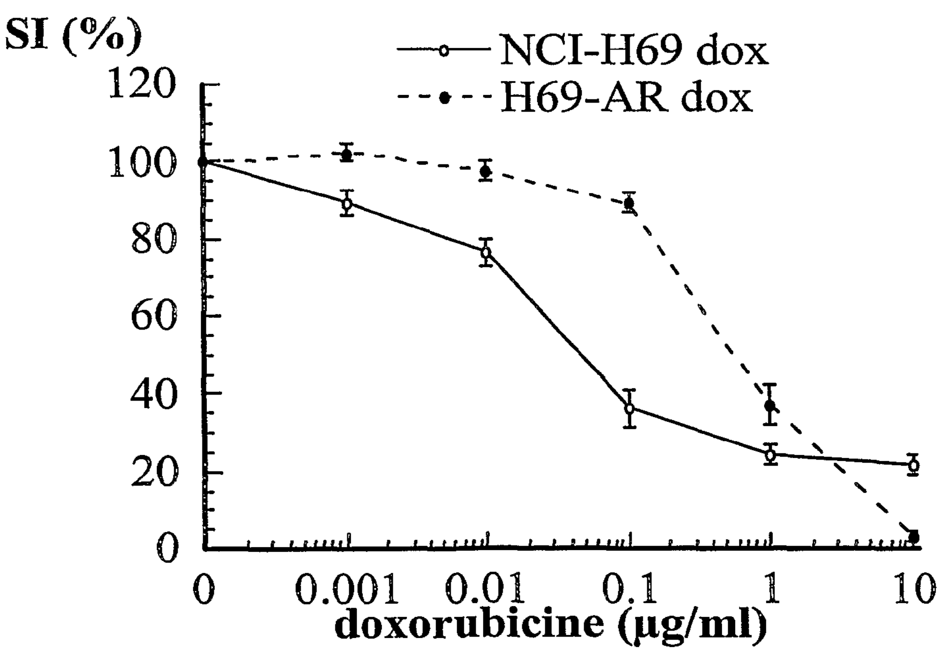
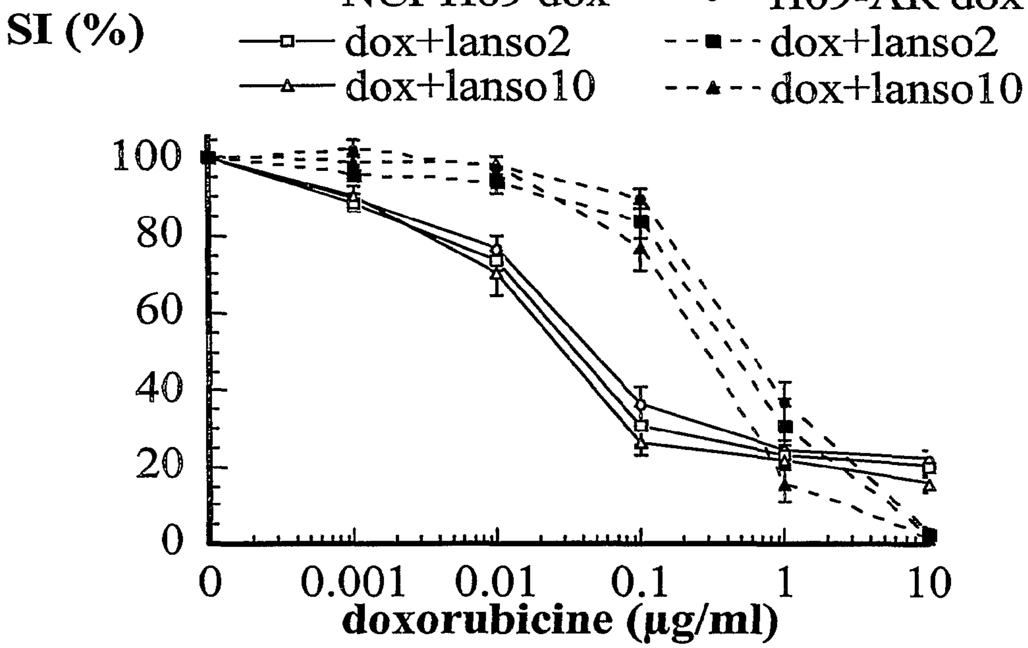


Fig. 3 cont.

3e.



3f.

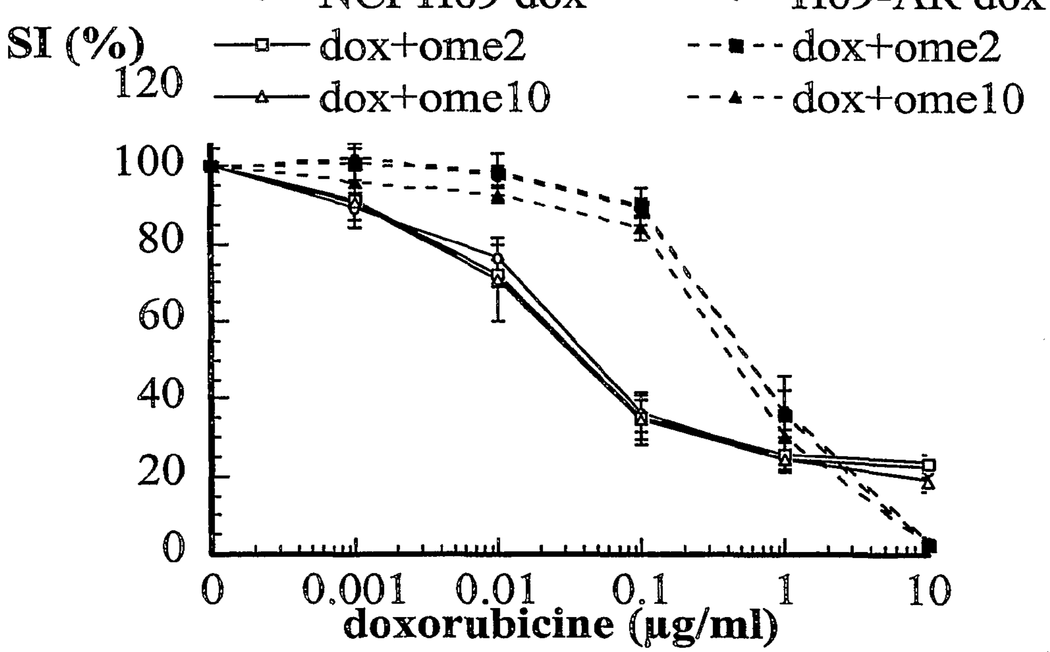


Fig. 3 cont.

3g.

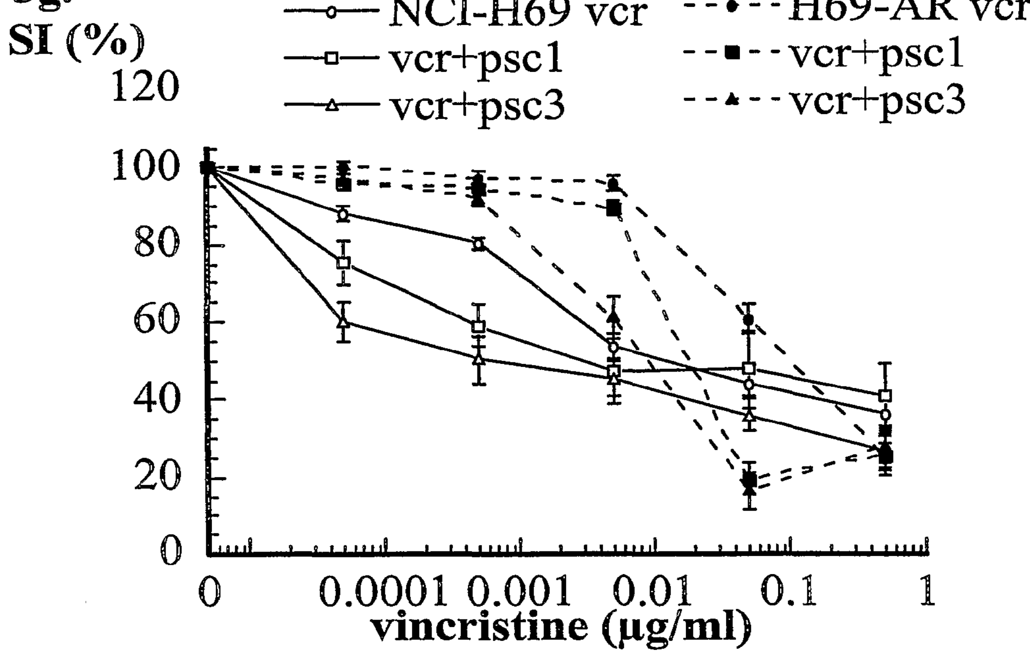

SI (%)
NCI-H69 vcr
vcr+psc1
vcr+psc3
H69-AR vcr
vcr+psc1
vcr+psc3
120
100
80
60
40
20
0
0
0.0001
0.001
0.01
0.1
1
vincristine (µg/ml)

Fig. 4a

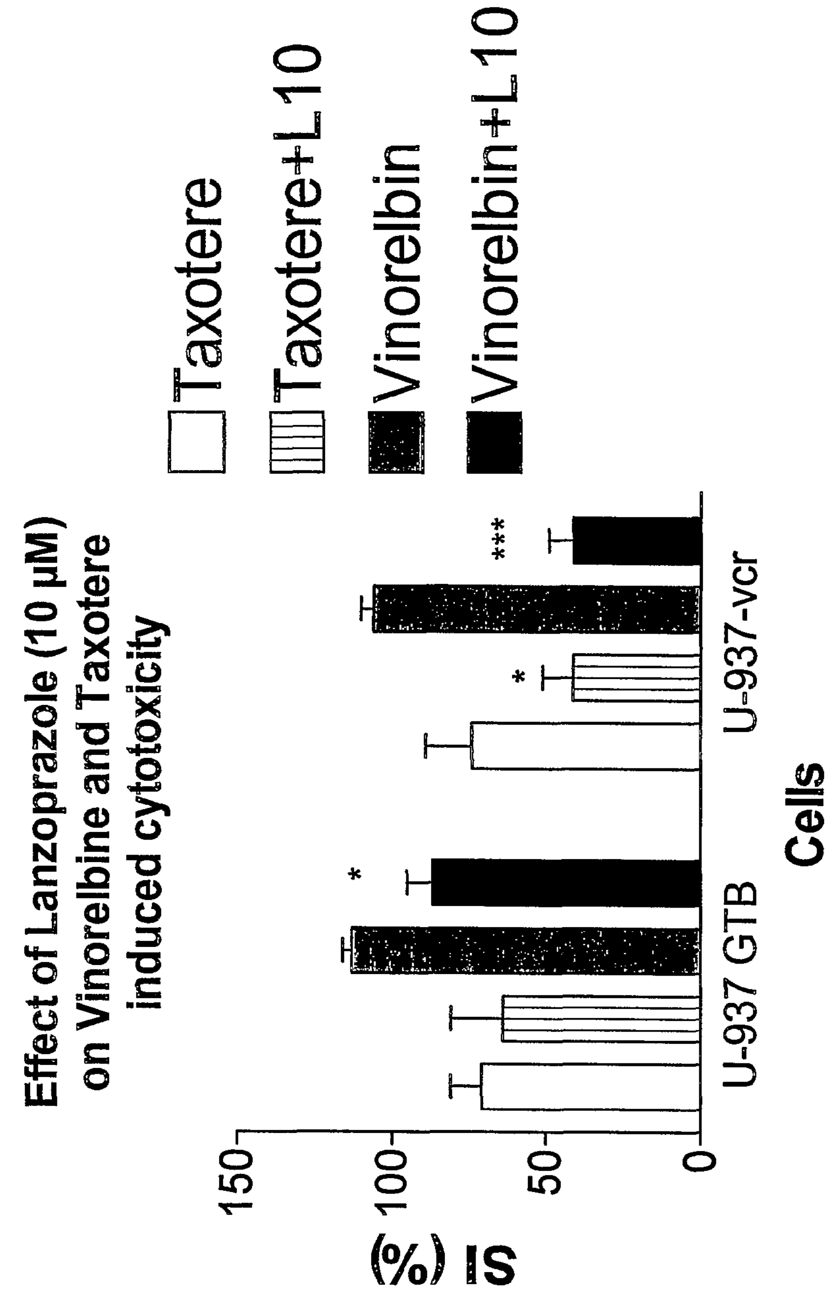

Effect of Lanzoprazole (10 µM) on Vinorelbine and Taxotere induced cytotoxicity
SI (%)
150
100
50
0
*
*
***
U-937 GTB
U-937-vcr
Cells
Taxotere
Taxotere+L10
Vinorelbin
Vinorelbin+L10

Fig. 4b

Effect of Lanzoprazole on Vinorelbin (Vrb) and Taxotere (Taxt) induced cytotoxicity in U-937 GTB and U-937-vcr cells (n=6)

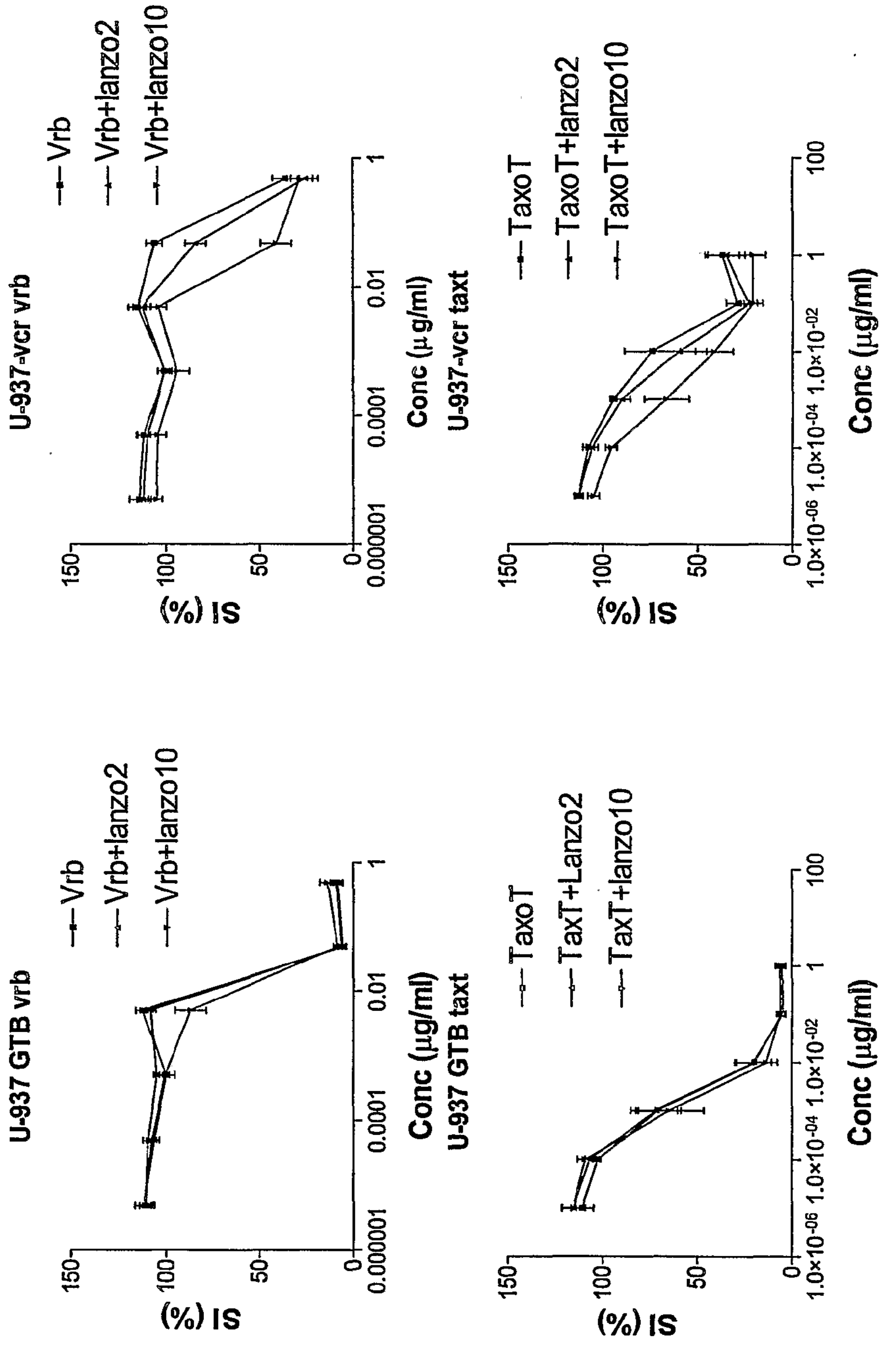


Fig. 5a

A.

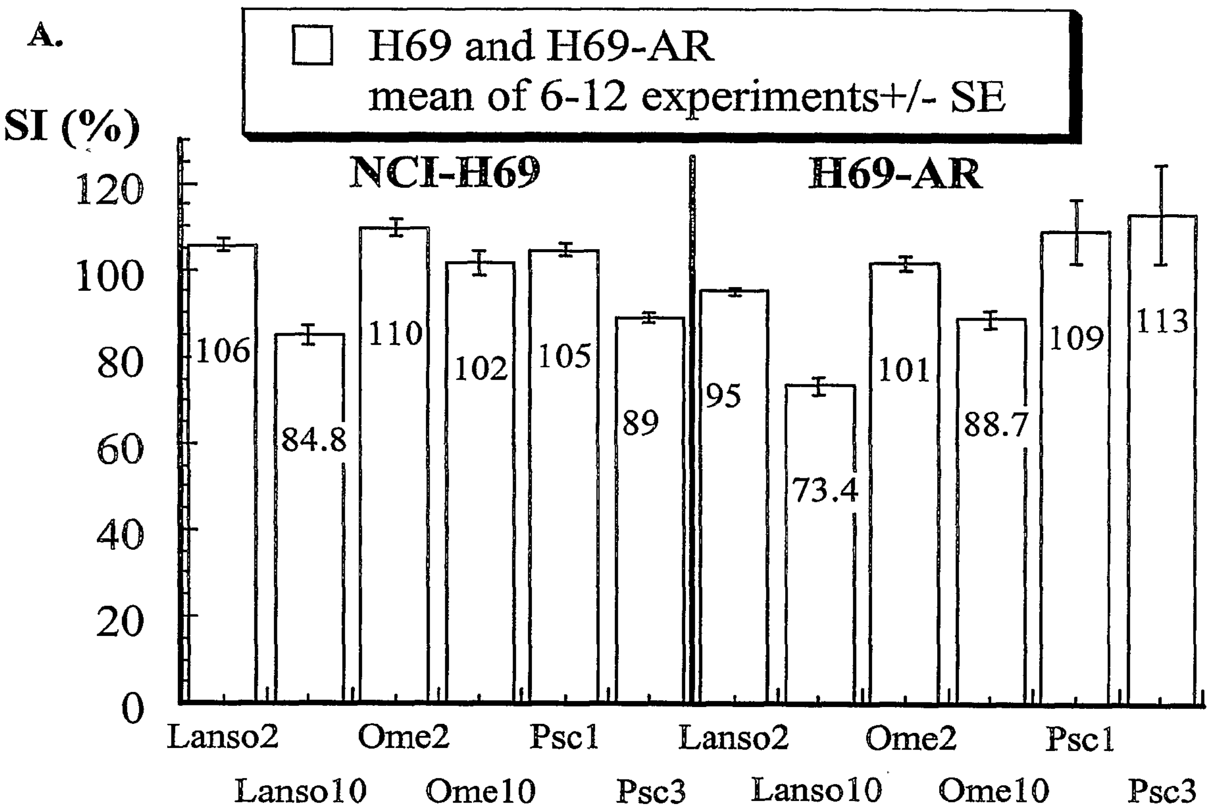

H69 and H69-AR
mean of 6-12 experiments+/- SE
SI (%)
NCI-H69
H69-AR
120
100
80
60
40
20
0
106
84.8
110
102
105
89
95
73.4
101
88.7
109
113
Lanso2
Lanso10
Ome2
Ome10
Psc1
Psc3
Lanso2
Lanso10
Ome2
Ome10
Psc1
Psc3

Fig. 5b

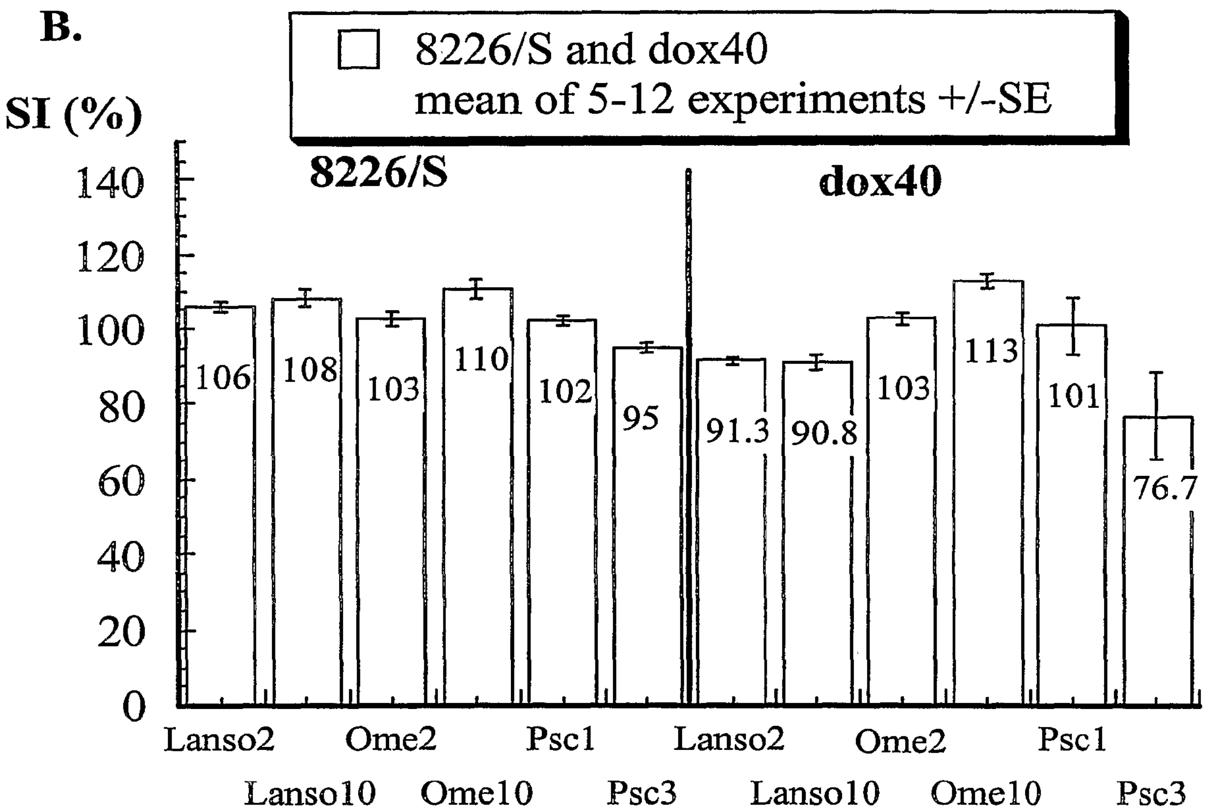

B.
8226/S and dox40
mean of 5-12 experiments +/-SE
SI (%)
8226/S
dox40
140
120
100
80
60
40
20
0
106
108
103
110
102
95
91.3
90.8
103
113
101
76.7
Lanso2
Lanso10
Ome2
Ome10
Psc1
Psc3
Lanso2
Lanso10
Ome2
Ome10
Psc1
Psc3

Fig. 5c

C.

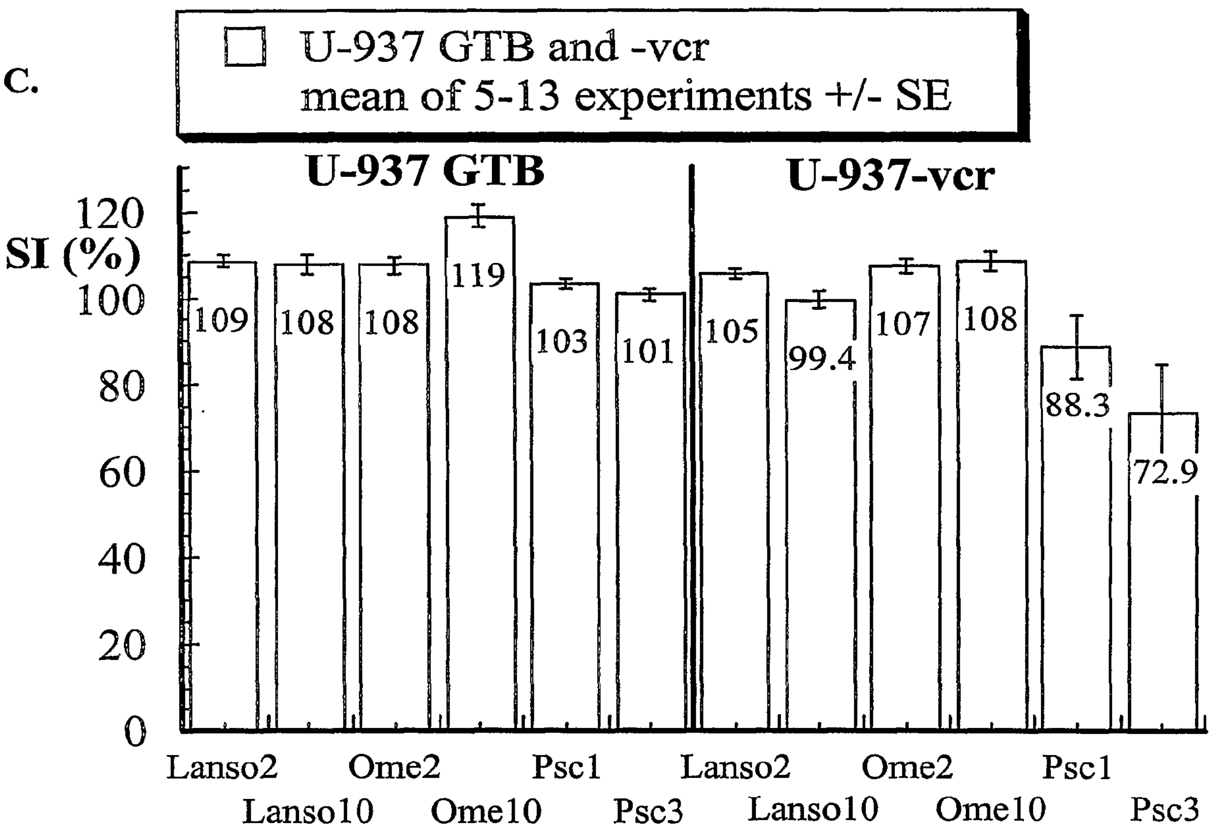

U-937 GTB and -vcr
mean of 5-13 experiments +/- SE
U-937 GTB
U-937-vcr
SI (%)
120
100
80
60
40
20
0
109
108
108
119
103
101
105
99.4
107
108
88.3
72.9
Lanso2
Lanso10
Ome2
Ome10
Psc1
Psc3
Lanso2
Lanso10
Ome2
Ome10
Psc1
Psc3

Fig. 6

Mean % inhibition of 5 polymerisation assays

CQ concentration/ml	0.5 µg	0.25 µg	0.125 µg	63 ng	32 ng	16 ng	8 ng	4 ng	2 ng
CQ	99	99	96	70	27	14	7	5	4
CQ+ Lanzo 20µg/ml	100	100	99	77	59	55	56	54	54
CQ+ Lanzo 10µg/ml	99	99	95	71	41	32	29	31	30
CQ+ Lanzo 5µg/ml	100	100	96	70	38	25	18	19	17
CQ+ Lanzo 2µg/ml	99	99	93	57	30	16	14	16	18

Lanzo 20µg/ml	53
Lanzo 10µg/ml	22
Lanzo 5µg/ml	4
Lanzo 2µg/ml	0

DMSO 10µg/ml	0
DMSO 20µg/ml	0

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 02/00678

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A61K 31/4439, A61K 31/4184, A61P 43/00 // C07D 401/12
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CHEM. ABS DATA, BIOSIS, EMBASE, MEDLINE, EPO-INTERNAL, WPI DATA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Helicobacter, Volume 4, No. 2, 1999, Matthias Trautmann et al: "Combined Activity of Azithromycin and Lansoprazole Against Helicobacter pyroli", pages 113-120, abstract, discussion -- --------	1-3,12-18

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 Sept 2002	18 -09- 2002
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Per Renström/EÖ Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE02/00678

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 17
because they relate to subject matter not required to be searched by this Authority, namely:
see next sheet

2. ☒ Claims Nos.: 1 and 3-18
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see next sheet

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3, 4-7 and 18 partly

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Box I.1

Claim 17 relates to a method of treatment of the human or animal body by surgery or by therapy/a diagnostic method practised on the human or animal body/Rule 39.1(iv). Nevertheless, a search has been executed for this claim. The search has been based on the alleged effects of the compound/composition.

Box I.2

The present claims 1 and 3-18 relate to uses, methods and compositions defined by reference to a desirable property of the compounds to be used, namely that they should be proton pump inhibitors. The claims cover uses, methods and compositions involving all compounds having this property, whereas the application provides support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds.

Independent of the above reasoning, the claims 1 and 3-18 also lack clarity (Article 6 PCT). An attempt is made to define the compounds by reference to a result to be achieved. This lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Specifically, the term "proton pump inhibitor(s)" apparently relates to a very large amount of different compounds, which do not necessarily have to be defined as proton pump inhibitors, thus rendering it impossible to perform a complete search.

Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to uses of the compounds mentioned claim 2, i. e. omeprazole, esomeprazole, lansoprazole, rabeprazole and pantoprazole.

Box II

According to PCT, Article 34 (3) (a-c) and PCT, Rules 13.1 - 13.2, an international application shall relate to one invention only or to a group of inventions linked by one or more of the same or corresponding "special technical features", i.e. features that define a contribution which each of the inventions makes over the prior art. Since the unifying technical feature, or general inventive concept, of the invention according to the present claims - i.e. the use of proton pump inhibitors for the production of a drug for treatment or prevention of a MDR (multidrug resistance) condition - does not make such a contribution over the discovered prior art (the proton pump inhibitor lansoprazole is known to counteract/prevent resistance against antibiotics), the application is found *à posteriori* to contain the following subjects, each falling under its own restricted inventive concept:

1. The invention according to claims 1-3, 4-7 and 18 partly, wherein the MDR condition is associated with cancer treatment with cytostatics/anti-neoplastic agents.

2. The invention according to claims 1-3, 8-11 and 18 partly, wherein the MDR condition is associated with treatment of a parasitic infection with an antiparasitic agent.

3. The invention according to claims 1-3, 12-16 and 18 partly, wherein the MDR condition is associated with treatment of a bacterial infection with an antibacterial agent.

Consequently, the present application lacks unity of invention. The search has been carried out only for the first invention, namely the invention according to claims 1-7 and 18 partly.